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(54) Title: LYTIC PEPTIDES, USE FOR GROWTH, INFECTION AND CANCER

(57) Abstract

Novel synthetic lytic and proliferative peptides were designed and constructed to encompass the structural features associated with lytic and proliferative activity. These compounds, along wih the human β fibrin signal peptide share structural and functional properties of the known lytic peptides. These peptides are effective agents in the treatment of microbial infections including gram negative and gram positive bacteria, fungus, virus, yeast, and protozoa, in the lysis of cancer cells, and in the proliferation of fibroblast and lymphocytes. Additional functions include synergy and use as general adjuvants and in the enhancement of wound healing.

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" Lytic Peptides, use for Growth, Infection and Cancer "

SPECIFICATION

Field of the Invention

The present invention relates to novel lytic peptides, their use in methods for inhibiting eucaryotic pathogens, cancer cells and intracellularly infected cells, their use in methods for stimulating the proliferation of fibroblast and lymphocytes, and in the process of wound healing. More particularly, this invention relates to the inhibition of such pathogens, cancers and infected cells, and the stimulation of fibroblast and lymphocytes in mammals and other higher animals. The present invention also relates to the use of lytic peptides as general adjuvants.

Background of the Invention

Many diseases of procaryotic origin, i.e. caused by pathogenic bacteria, are known. Such diseases are in general more easily treated than those of eucaryotic origin because of the marked differences between the invading procaryotes and the eucaryotic cells of the host. Thus, because of the differences between bacterial cells and those of the host, many antibiotics are known to specifically inhibit the invading bacteria without significant adverse effects on the host. In contrast, it has generally been more difficult to treat diseases of nonbacterial origin, such as malaria, sleeping sickness and Chagas' disease.

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The property of certain peptides to induce lysis of procaryotic microorganisms such as bacteria are known. For example, U.S. patents 4,355,104 and 4,520,016 to Hultmark et al describe the bacteriolytic properties of some cecropins against Gram-negative bacteria. Quite interestingly, the cecropins described in the Hultmark et al. patents were not universally effective against all Gram-negative bacteria. For example, the cecropins described therein lysed _Serratia marcescens_ D61108, but not _Serratia marcescens_ D611. Moreover, cecropins have heretofore been reported to have no lytic activity towards eucaryotic cells such as insect cells, liver cells and sheep erythrocytes, as reported in the Hultmark patents; International Patent Publication WO/8604356; Andreu et al _Biochemistry_, vol. 24, pp. 1683-88 (1985); Boman et al, _Developmental and Comparative Immunology_, vol. 9, pp. 551-558 (1985); and Steiher et al., _Nature_, vol. 292, pp. 246-248 (1981).

Other lytic peptides heretofore known include, for example, the sarcotoxins and lepidopterans. Such peptides generally occur naturally in the immune system of _Sarcophaga peregrina_ and the silkworm, lepidopteran, respectively, as reported in Nakajima et al, _The Journal of Biological Chemistry_, vol. 262, pp. 1665-1669 (1987) and Nakai et al, _Chem. Abst._ 106:214351w (1987).

The mechanism of action of the lytic peptides in the immune systems in which they occur is not entirely clear. There must, of course, be some aspect of the mechanism which regulates the specificity of the lytic peptides for invading pathogens among the cells of the host organism which must generally be preserved from lysis. For example, human complement fixation involves antibodies which are generally specific for certain antigens expressed by the invading pathogen. The

activated components of complement attack the membrane of the invading cell to which they are bound by the antigen-antibody reaction to produce circular lesions which are probably formed as a result of insertion of the C9 protein into the membrane. The more primitive mechanisms involved in insect immunology are less specific, but the peptides involved apparently do not significantly lyse the host cells.

There are many differences between the membranes of different types of cells which can affect their susceptibility to lysis by the various lytic peptides. As suggested above, for example, some proteins are capable of lysing only cells expressing an appropriate antigen for the antibody associated with such protein. Thus, it is not surprising that the less specific lytic peptides such as cecropins are more capable for lysing procaryotes than the eucaryotic cells of the insect.

Gram-positive procaryotes generally have a thicker cell wall than Gram-negative ones. Also, Gram-positive cell membranes have a cytoplasmic membrane and a cell wall containing mostly peptidoglycans and teichoic acids, whereas Gram-negative cell membranes have an inner cell wall consisting entirely of peptidoglycan and associated proteins surrounded by an outer cell wall comprised of lipid, lipopolysaccharide and protein. In contrast, eucaryotic cells generally have a plasma membrane comprising a lipid bilayer with proteins and carbohydrates interspersed therein, and also have organelles with their own membrane systems, but generally do not have an outer cell wall. It is readily appreciated that the considerable variation of membrane structures among bacteria (procaryotes) accounts for considerable variation in their susceptibility to lysis by the various insect immune proteins.

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The variation of membrane structures among
eucaryotes is also considerable, but these membranes
generally comprise phospholipid molecules in a bilayer
arrangement with a thickness of about 50Å. The
hydrophilic portion of the phospholipid is generally
oriented to the exterior and interior surfaces of the
membrane, while the hydrophobic portions are generally
found in the interior region of the membrane between
the hydrophilic surfaces. As reported in Nakajima et
al, the presence of cholesterol and the asymmetric
distribution of phospholipids in the cytoplasmic
membrane of eucaryotic cells may explain the selective
toxicity of sarcotoxin to bacteria. Since cholesterol
causes condensation of the phospholipid bilayers, it
can hinder the penetration of lytic peptides into the
cytoplasmic membrane of eucaryotic cells. Similarly,
the predominance of neutral phospholipids in the outer
monolayer of eucaryotic membranes would result in less
affinity to positively charged lytic peptides such as
cecropin and sarcotoxin than acidic phospholipids
generally located on the cytoplasmic side of the
membrane.

Eucaryotic cells have a high degree of internal
organization conferred by a complex matrix of proteins
known as the cytoskeleton. Many of the proteins of the
cytoskeletal matrix are anchored in the cell membrane.
The degree of an intact cytoskeletal organization may
determine the ability of lytic peptide to lyse the
cell.

A number of the antibacterial polypeptides have
been found to be useful when the genes encoding
therefor are incorporated into various plant species.
Particularly, when introduced into the plant genome by
means of an Agrobacterium plasmid vector, the
antibacterial polypeptide-encoding genes produce plant

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species much more resistant to certain bacterially
induced disease conditions and plant pathogens. Such
antibacterial polypeptides and the transformation of
plants with genes encoding therefor are described in
aforementioned U.S. patent application Serial No.
889,225.

Polynucleotide molecules expressible in a given
host and having the sequence araB promoter operably
linked to a gene which is heterologous to such host are
also known. The heterologous gene codes for a
biologically active polypeptide. A genetic construct
of a first genetic sequence coding for cecropin
operably linked to a second genetic sequence coding for
a polypeptide which is capable of suppressing the
biological effect of the resulting fusion protein
towards an otherwise cecropin-sensitive bacterium is
also described in International Publication WO86/04356,
July 31, 1986.

The Hultmark et al patents mentioned above also
mention that there are not known antibodies to
cecropin, indicating a wide acceptability for human and
veterinary applications, including one apparently
useful application for surface infections because of
the high activity against pseudomonas. Similarly, EPO
publication 182,278 (1986) mentions that sarcotoxins
may be expected to be effective in pharmaceutical
preparations and as foodstuffs additives, and that
antibacterial activity of sarcotoxin can be recognized
in the presence of serum. Shiba, Chem. Abstr. 104:
230430K (1985) also mentions preparation of an
injection containing 500 mg lepidopteran, 250 mg
glucose in injection water to 5 ml.

Several analogs of naturally-occurring cecropins,
sarcotoxins and lepidopterans have been reported. For
example, it is reported in Andreu et al, Proc. Natl.

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Acad. Sci. USA, vol. 80, pp. 6475-6479 (1983) that
changes in either end of the amino acid sequence of
cecropin generally result in losses in bactericidal
activity in varying degrees against different bacteria.
It is reported in Andreu et al (1985) mentioned above
that Trp is clearly important for bactericidal activity
of cecropin, and that other changes in the 4, 6 or 8
position have different effects on different bacteria.
From the data given in Table II at page 1687 of Andreu
et al (1985), it appears that almost any change from
natural cecropin generally adversely affects its
bactericidal activity.

Cecropin is defined in International Publication
WO86/04356 to include bactericidally active
polypeptides from any insect species and analogs,
homologs, mutants, isomers and derivatives thereof
having bactericidal activity from 1% of the naturally-
occurring polypeptides up to 100 times or higher
activity of the naturally-occurring cecropin. Other
references generally discuss the effects of the -helix
conformation and the amphiphilic nature of cecropin and
other lytic peptides.

It is known that lysozyme and attacins also occur
in insect homolymph. For example, it is reported in
Okada et al, Biochem. J., vol. 229, pp. 453-458 (1985)
that lysozyme participates with sarcotoxin against
bacteria, but that the bactericidal actions are
diverse. Steiner et al mentioned above suggests that
lysozyme plays no role in the antibacterial activity of
insect hemolymph other than to remove debris following
lysis of bacteria by cecropin. Merrifield et al,
Biochemistry, vol. 21, pp. 5020-5031 (1982) and Andreu
et al (1983) mentioned above state that cecropin
purified from insect hemolymph may be contaminated with
lysozyme, but demonstrate that the synthetically

prepared cecropin is as bactericidally active as
purified cecropin from insect hemolymph.

The analogs of lytic peptides known at this time
are primarily conservative, point mutations of amino
acids which tend to disrupt the activity of the lytic
peptide. The analogs of cecropin described in the
previous patent application, U.S. Patent 102,175, SB-
37 and Shiva-1 are synthetic analogs which preserve the
lytic activity of the natural cecropins. It would be
of great utility to understand the structural
properties of the known lytic peptides which relate to
their diverse functional properties. With such
understanding, it would then be of great utility to
design and synthesize polypeptides with the specific
activities desired.

Polypeptides having high lytic activity against
gram positive and gram negative bacteria, fungus,
yeast, protozoa, and plant or animal cells infected
with pathogens, would be of great medical and
agricultural importance, particularly if such
polypeptides were economically and easily produced.

Polypeptides having high lytic activity against
cells infected with pathogens or cancerous cells would
be of great therapeutic importance in the treatment of
animals.

Polypeptides having high proliferative activity
would be advantageous in stimulating the immune system
and in wound healing.

Summary of the Invention

The present invention discloses synthetic lytic
peptides, particularly designed and constructed to
encompass those structural properties believed to be
associated with lytic and proliferative function:
aligned amphipathic α-helical conformation, with

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positive charge density. The synthetic lytic peptides of this invention are designed to function in the treatment of plants and animals against microbial infections including bacterial, yeast, fungal, viral

5   and protozoan infections. These lytic peptides are also designed to function in the lysis of cancer cells.

The present invention also discloses the human Beta fibrin signal peptide and analogs as lytic peptides sharing similar structural and functional

10   properties of the lytic peptides.

The present invention also discloses synthetic lytic peptides which stimulate cell growth and assist in the process of wound healing.

The synthetic lytic peptides of this invention

15   also function synergistically with conventional therapeutic agents such as antibiotics and anti-cancer treatments, and may be used as general adjuvants.

Accordingly, the invention provides a method for lysing microbial and eucaryotic cells which includes

20   contacting the cells with a lytic peptide in an amount effective to lyse the cells. The cells include gram positive and gram negative procaryotic microorganisms, lymphomas, leukemias or carcinomas, or eucaryotic cells infected with an intracellular pathogenic

25   microorganism. The lytic peptide has from about 20 to about 40 amino acids, at least a portion of which are arranged in an aligned amphiphilic α-helical conformation. The peptide has a substantially hydrophilic head with a positive charge density and a

30   substantially hydrophobic tail. The conformation has a predominately hydrophobic face along the length of the conformation and a predominately hydrophilic face opposed therefrom.

The invention also provides a method for

35   selectively lysing eucaryotic cells in the presence of

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cells which are not lysed. The method includes contacting target eucaryotic cells in the presence of non-target cells with a selectively lytic, free peptide in an amount effective to lyse the target cells. The target cells are eucaryotic microorganisms, lymphomas, leukemias or carcinomas, or eucaryotic cells infected with an intracellular pathogenic microorganism. The lytic peptide contains from about 20 to about 40 amino acids, at least a portion of which are arranged in an aligned amphiphilic α-helical conformation with a positive charge density.

In another aspect, the invention provides a method for lysing eucaryotic microorganisms which includes contacting the eucaryotes with an amount of a lytic peptide effective to lyse the microorganisms. The peptide includes from about 20 to about 40 amino acids, at least a portion of which are arranged in an aligned α-helical conformation with a positive charge density.

In still another aspect, the invention provides a method for lysing cancer cells. The method includes contacting lymphoma, leukemia or carcinoma cells with an effective amount of a lytic peptide to lyse the cells. The peptide has from about 20 to about 40 amino acids at least a portion of which are arranged in an aligned α-helical conformation with a positive charge density.

Further, the invention provides a method for selectively lysing infected eucaryotic cells. The infected eucaryotic cells are infected with an intracellular pathogenic microorganism, such as, for example, virus, bacteria, fungi or protozoa. The method includes contacting the infected and uninfected cells with a selectively lytic, free peptide in an amount effective to selectively lyse the infected cells

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and leave the uninfected cells substantially free of lysis.

Still further, the invention provides a method for inhibiting eucaryotic cells in a higher animal. The method includes introducing a selectively lytic, free peptide into the higher animal in an amount effective to inhibit therein eucaryotic cells such as eucaryotic microorganisms, mammalian lymphomas, leukemias or carcinomas, or cells infected with an intracellular pathogenic microorganism.

Still further, the invention provides a method for stimulating the proliferation of normal mammalian fibroblast and lymphocytes which includes contacting the fibroblast or lymphocytes with a stimulating peptide of approximately 15 to 40 amino acids in an amount effective to stimulate the proliferation thereof. There is also provided a method for stimulating the proliferation of normal fibroblast and lymphocytes in a mammal which includes introducing a stimulating peptide of approximately 15 to 40 amino acids into a mammal in an amount effective to stimulate the proliferation of fibroblast or lymphocytes therein. This stimulating peptide contains an aligned amphipathic alpha-helical conformation with a positive charge density.

Also provided is a method for enhancing the process of wound healing in animals which includes contacting the wound with a stimulating peptide of 15 to 40 amino acids in an amount effective to enhance the healing of the wound.

In other aspects, the invention provides a synergistic composition containing lytic peptide and another therapeutic or diagnostic agent, in an amount sufficient to facilitate activity of the agent in cells.

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Brief Description of the Drawings

Figure 1 illustrates the amphipathic alpha helical conformation of cecropin B; its hydrophobic and hydrophilic distribution; and its positive charge distribution.

Figure 2 illustrates the distribution of hydrophobicity and hydrophilicity along the alpha helix and the positive charge distribution for each class of synthetic lytic peptides.

Figure 3 lists the amino acid sequences of the synthetic lytic peptides.

Figure 4 illustrates the distribution of hydrophobic and hydrophilic amino acid residues and the location of the positively charged amino acid residues for each of the synthetic lytic peptides.

Figure 5 illustrates the Edmunson helical wheels of the synthetic lytic peptides.

Figures 5, 6 and 7 illustrate Edmunson helical wheels for the lytic peptides.

Figures 8 show photographs of rats burned and treated with lytic peptides to enhance wound healing.

Detailed Description of the Preferred Embodiments

It has been found that lytic peptides having about 20-40 amino acids are capable of lysing or otherwise inhibiting bacterial or eucaryotic cells which are involved with the disease or illness of plants and animals. Such eucaryotic cells include, for example, protozoa, fungi, algae, cancer cells such as lymphomas, leukemias and carcinomas, and cells infected with intracellular fungi, bacteria, protozoa or viruses. On the other hand, certain lytic peptides of 15 to 40 amino acids have also been found to stimulate the

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proliferation of lymphocytes an fibroblast; and enhance wound healing.

As used herein, the term "lytic peptide" includes any polypeptide which lyses the membrane of a cell in an _in vivo_ or _in vitro_ system in which such activity can be measured. Suitable lytic peptides used in the present invention have lytic activity toward one or more bacterial cells, eucaryotic cells such as protozoa, fungi, helminths, leukemias, lymphomas or carcinomas, or cells infected by intracellular pathogens. Preferred lytic peptides have from about 20 to about 40 amino acids, while preferred growth-promoting peptides have about 15-40 amino acids, at least a portion of these amino acids are arranged in an aligned α-helical conformation having a substantially hydrophilic head with a positive charge density, a substantially hydrophobic tail, and a part of opposed faces along the length of the helical conformation, one such face being predominantly hydrophilic and the other being predominantly hydrophobic. The head of this conformation may be taken as either the amine terminus end or the carboxy terminus end.

The structural features of the known lytic peptides thought to be associated with lytic function are aligned amphipathy and a substantial positive charge density.

The structural features of cecropin B are illustrated in Figure 1. Figure 1A demonstrates the alpha helical configuration of cecropin B, and the distribution of hydrophobic and hydrophilic amino acid residues which form its aligned amphipathic region. Figure 1B illustrates an Edmundson wheel diagram of cecropin B, indicating an internal ring of 18 amino acids representing the amino-terminal portion of the helix and the external ring of 18 amino acids

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representing the carboxy-terminal portion of the molecule. The amino acids conferring positive charge to the molecule are represented by the symbol, "+".

Figure 1C illustrates the length of the alpha helix, divided to represent two longitudinal faces of the molecule and divided again into its amino and carboxy terminal halves. The shaded portion of the figure represents the substantially hydrophilic region of the molecule while the open areas represent hydrophobic regions.

Thus Figure 1C represents the aligned amphipathic nature of the amino terminal half of the cecropin B molecule.

An "aligned amphipathic" α-helix is defined herein as that distribution of hydrophobic and hydrophilic amino acid residues along opposing faces of the alpha-helix which results in one face of the helix being predominantly hydrophobic and the opposite face being predominantly hydrophilic. Hydrophilic amino acids are defined as those having a transfer free energy of <1.0 Kcal/mole. The following table lists the free energies of the 20 natural amino acids:

HYDROPHOBICITY/HYDROPHILICITY

Amino Acid	Transfer Free Energy (Kcal/mole)	
F	3.7	Phenylalanine
M	3.4	Methionine
I	3.1	Isoleucine
L	2.8	Leucine
V	2.6	Valine
C	2.0	Cysteine
W	1.9	Tryptophan
A	1.6	Alanine
T	1.2	Threonine

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G	1.0	Glycine

S	0.6	Serine
P	-0.2	Proline
Y	-0.7	Tyrosine
H	-3.0	Histidine
Q	-4.1	Glutamine
N	-4.8	Asparagine
E	-8.2	Glutamate
K	-8.8	Lysine
D	-9.2	Aspartate
R	-12.2	Arginine

D.M. Engelmann et al, Annual Review of Biophysical Chemistry, Vol. 15 330 (1986).

A "selectively lytic peptide" is a lytic peptide which preferentially lyses target cells in a system comprising both target and non-target cells, wherein the target cells are selected from bacteria, protozoa, fungi, mammalian leukemias, lymphomas and carcinomas and eucaryotic cells infected by intracellular pathogens. The selectively lytic peptides used in the present methods are preferably "free peptides," i.e. undirected in action by an antibody and otherwise unbound or unfused to another molecular fragment which adversely affects its lytic activity.

Suitable lytic peptides generally include cecropins such as cecropin A, cecropin B, cecropin D, and lepidopteran; sarcotoxins such as sarcotoxin IA, sarcotoxin IB, and sarcotoxin IC; magainin 1 and magainin 2 and other polypeptides obtainable from the hemolymph of any insect species which have lytic

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activity against bacteria similar to that of the
cecropins and sarcotoxins. Melittin is generally not
considered suitable because of its extreme toxicity.
It is also contemplated that lytic peptides may be
obtained as the lytically active portion of larger
peptides such as attacins; lysozymes; certain phage
proteins such as S protein of phage, E protein of
PHIX 174 phage and P13 protein of P22 phage; and C9
protein of human complement. As used herein, classes
of lytically active peptides such as, for example,
"cecropins," "sarcotoxins" and "phage proteins," and
specific peptides within such classes, are meant to
include the lytically active analogues, homologues,
mutants or isomers thereof unless otherwise indicated
by context.

The human beta fibrin signal peptide shares common
features of the known lytic peptide including
significant aligned amphipathy and associated high
positive density. The human beta fibrin signal peptide
has now been found to share common functional
properties with the lytic peptides including
antibacterial and anti-cancer properties as well as the
proliferative effects.

Novel synthetic lytic peptides were designed and
constructed to incorporate and improve those structural
features associated with lytic and proliferative
activities. The essential features of these peptides
include a minimal length of approximately 19 amino
acids; an helical conformation; exhibiting significant
aligned amphipathy and a positive charge density.

The preferred peptides are those of 15-40 amino
acids in which one or more sets of 18 linear amino
acids spaced 20° apart about the axis of the helix
exhibits 60-90% aligned amphipathy.

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The amino acid sequence of the preferred peptides includes greater than or equal to four positively charged amino acids, such as lysine, arginine, histidine, ornithine and citrulline.

The lytic peptides of the present invention may be grouped into five structural classes according to the location of their aligned amphipathy as illustrated in Fig. 2.

Specific peptides each of these classes include the amino acid sequences are listed in Fig. 3, the hydrophobic/hydrophilic table in figure 4, and Edmundson helical wheels shown in figures 5, 6 and 7.

Class No. 1 - Cecropin Analogs:

Synthetic lytic peptides in the group of cecropin analogs are comprised of peptides having approximately 23 to 38 amino acids. These peptides have an aligned amphipathic region and a positive charge density distributed over the amino-terminal half of the molecule, and therefore only one set of 18 amino acids exhibiting aligned amphipathy.

The Cecropin-like class of synthetic lytic peptides comprises members such as SB37, LSB37 and LSB*37 which differ only slightly in amino acid sequence from cecropin B, and other members such as the Shiva series which differ greatly from cecropin B (approximately 50% amino acid homology). All members of this class do share a high degree of homology in hydrophobicity, amphipathy, and charge distribution.

Class No. 2 - Manitou Series:

The lytic peptides classified in the Manitou series are cecropin-like analogs in that their positive charge density and aligned amphipathy is distributed over the amino terminal half of the molecule. However,

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included in the Manitou series is the human beta-fibrin
signal peptide, which has little amino acid homology
with cecropin.

Class No. 3 - Anubis Series:

5   The synthetic lytic peptides classified in the
Anubis series constitute simple, economic and easily
produced peptides comprised of repeats of 3 to 5 simple
amino acids chosen from the group comprising: alanine,
leucine, glutamine, phenylalanine and lysine. It is
10   anticipated that other amino acids may also be used,
providing the essential structural properties of the
polypeptide is maintained. These peptides have little
or no amino acid homology to the natural magainins,
however, the aligned amphipathy and positive charge
15   density is highly homologous. The peptides of the
Anubis series have a total length of approximately 21
to 38 amino acids, and are comprised of two sets of 18
linear amino acids exhibiting aligned amphipathy.

Class No. 4 - Hecate Series:

20   The Hecate Series is also comprised of simple
combinations of 3 to 5 amino acids. The peptides in
this series have varied lengths of amino acids forming
the hydrophilic tail of the molecule.
        The lytic peptides in the Hecate series are
25   comprised of peptides having an aligned amphipathic
region and positive charge density distributed over the
carboxy terminal half of the molecule in a manner
homologous to the lytic peptide melittin, but with
little or no amino acid homology to the known peptide.
30   The lytic peptides in this series are of approximately
23 to 38 amino acids in length, with the carboxy
terminal amino acid designated as number 1. These

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peptides contain one set of 18 linear amino acids
exhibiting aligned amphipathy.

Class No. 5 - Vishnu Series:

The Vishnu Series is comprised of the amino-
terminal half of the cecropin-like analogs. The amino
acid sequence is identical to the first 19 amino acids
of these peptides. Vishnu peptides are "halfmers" of
the cecropin analogs and have little or no lytic
activity.

It is contemplated that the shortened length of
the molecules may not enable lytic function, as the 19
amino acid helix may not span the cell membrane.

The synthetic peptides of the Vishnu series are
comprised of the amino terminal half of the cecropin
analogs. These peptides are approximately 19 amino
acids in length and have complete aligned amphipathy,
no hydrophobic tail, and have a positive charge density
distributed throughout the molecule. There are no
known natural analogs to this series.

While the specific amino acid structure of the
lytic peptides was found not to be critical for lytic
function, the hydrophobic/hydrophilic amino acid and
positively charged amino acid distribution was found to
be highly significant.

The hydrophobic/hydrophilic amino acid and
positively-charged amino acid distribution of synthetic
lytic peptides and their comparison to cecropin B is
illustrated in Figure 4.

It is anticipated that conservative substitutions
for these amino acids may include D and L forms and
synthetic amino acids such as ornithine, citrulline,
and homoserine, provided that the essential structural
features are maintained.

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The lytic peptides of the present invention are effective in lysing various types of bacterial and eucaryotic cells such as gram positive and gram negative bacteria, eucaryotic microorganisms, mammalian neoplastic cells and cells infected with intracellular pathogenic microorganisms. Eucaryotic microorganisms include, for example, fungi, yeasts, and protozoans such as sarcodina, mastigophora, ciliata and sporozoa. The lytic peptides are particularly effective against _Trypanosoma cruzi_ and _Plasmodium falciparum_ which are the causative agents of Chagas disease and malaria, respectively, and are also contemplated as being particularly effective against _Trypanosoma gambiense_ which is the causative agent of African sleeping sickness. The method of the present invention is useful to lyse or inhibit cancer cells such as lymphomas, leukemias and carcinomas, and particularly mammalian cancer cells of these types.

Suitable infected eucaryotes subject to lysis or inhibition according to the present method include cells infected with intracellular pathogenic microorganisms such as viruses, bacteria, fungi, or protozoans. Specific representative examples of such pathogens, the host cells of which are contemplated as suitable for lysis or inhibition according to the present method, include protozoa such as _P. falciparum_, _T. cruzi_, bacteria such as _Listeria monocytogenes_, _Brucella abortus_ and viruses such as parainfluenza, measles, HIV, and herpes simplex II. The method is particularly effective for the treatment of HIV and herpes virus-infected cells. Such pathogens grow or replicate within the infected cell and are generally protected from inhibition or lysis by the membrane of the host cell. However, the method of the present invention results in lysis of the host cell so that the

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intracellular pathogen is subsequently destroyed or subject to lysis or inhibition with the lytic peptides of the present invention or another inhibiting agent since it is no longer protected by the host cell membrane.

In accordance with the present method, the lytic peptide is used to lyse or otherwise inhibit eucaryotic cells by contacting the cells with the peptide. The amount of the lytic peptide necessary to induce cell lysis will usually be at least 0.1 μM, and preferentially 50-100 μM.

In a system in which the cells contacted with the lytic peptide are cultured or grown for obtaining biological or biochemical products therefrom, a cytoplasmic product may be desirably recovered from the lysed cells. For example, the recovery of a product such as interferon may be facilitated by treating cells producing the interferon product with a lytic peptide in combination with cytoskeletal formation inhibitors such as cytochalasin B, avoiding the use of detergents which complicates purification of the desired product.

In a preferred embodiment, the target cells to be lysed are lysed or otherwise inhibited in the presence of non-target cells. For example, the target cells may be an in vitro culture, mixture, or suspension, or may be target cells in a higher animal host, particularly chordate animals and chordate or nonchordate aquacultural animals, and especially mammals such as man. In such a situation, some degree of care is exercised in the selection of the lytic peptide and its concentration to avoid substantial lysis or inhibition of the non-target cells. For use in vivo, an amount of lytic peptide in the range of from about 1 mg/kg to about 100 mg/kg is usually sufficient to effect the desired inhibition and avoid substantial inhibition of

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the non-target or host cells, although this dosage may be increased or decreased, or repeated in a series of applications. The peptide may be introduced directly into the higher animal in any conventional manner, e.g. by injection of the peptide in a pharmaceutically acceptable carrier intramuscularly, subcutaneously, intravenously, or intraperitoneally, and preferably at or near the site of infection or cancer. Where there may be a relatively high incidence of target cells in a host, particularly in advanced stages of infection or cancer, additional caution should be exercised since rapid lysis of such a large number of target cells may induce a host reaction to the products of the lytic or inhibitory activity.

Although the present invention is not bound or limited by theory, it is believed that the membranes of the lower eucaryotes are generally subject to lysis by lytic peptides because of the differences in their membranes and cytoskeletal components. However, the membranes of normal cells of the higher eucaryotes somehow prevent lysis, possibly for example, by the ability of their well-developed cytoskeletons to quickly repair any membrane damage caused by the lytic peptide. On the other hand, higher eucaryotic cells with an aberrant cytoskeleton, such as neoplastic or infected cells, are generally unable to prevent lysis by the lytic peptide in the present method.

Quite unexpectedly, it has also been found that the lytic peptide can also stimulate the proliferation of fibroblast and mitogen activated lymphocytes by contacting the lymphocytes or fibroblast with an effective amount of a stimulating peptide. As used herein, the term "stimulating peptide" includes not only the preferred lytic peptides described hereinabove having from about 20 to about 40 amino acids, but also

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includes such peptides having fewer than 20 or more
than 40 amino acids containing an active form of the
portion of such lytic peptides inducing such
stimulation, whether or not they are lytically active.
Preferred stimulating peptides include peptides having
the first approximately 15-20 amino acids from the
amine terminus of lytic peptides such as cecropins and
sarcotoxins. In this sense, "stimulation" means an
enhancement of proliferation in any system in which it
can be observed or measured, and the stimulating
property of such peptides may or may not be related to
their lytic property. For convenience, reference is
made hereinbelow to cecropin by way of example with the
understanding that other stimulating peptides may be
used.

     Generally, lymphocytes must be activated by a
mitogen, or an antigen reactive with antibodies
expressed by the lymphocytes, before any stimulation
thereof by a stimulating peptide such as cecropin.
Mitogens include, for example, phytohemagglutinin,
pokeweed mitogen, concanavalin A, substances for which
lymphocytes have developed "memory" such as tetanus
toxoid, and the like. Thus, in one embodiment,
activated lymphocytes (e.g., lymphocytes in the
presence of a mitogen) or fibroblast are contacted with
a stimulating peptide in vitro to increase
proliferation thereof. In this manner, the production
of biological products by lymphocytes, for example,
such as interferon and interleukin-2, may be enhanced
by culturing the lymphocytes in the presence of, for
example, pokeweed mitogen and cecropin.

     In accordance with this embodiment, the
lymphocytes or fibroblast to be stimulated are
contacted with an effective amount of stimulating
peptide, generally an amount sufficient to provide a

concentration in the medium containing the lymphocytes
or fibroblast of from about 1 to about 50 μM. In a
preferred embodiment in which the stimulating peptides
are administered in vivo, contemplated dosages of the
peptides range from about one to about 10 mg/kg. The
peptide may be introduced into the animal to be treated
by injection in a suitable pharmaceutical carrier such
as saline solution, for example, intramuscularly,
subcutaneously, intravenously or intraperitoneally,
preferably at or near the site where such stimulation
is desired, e.g. a wound, graft, injury or infection.
The treatment may be repeated as necessary to sustain
the proliferative stimulation of the lymphocytes or
fibroblast.

Although the present invention is not bound or
limited by any theory, it is believed that the
stimulation of fibroblast and lymphocytes results from
the binding of the amphipathic, α-helical amine
terminus portion or end of the preferred lytic peptide
to lymphocyte and fibroblast cell membranes. No
binding activity is believed to be associated with the
generally hydrophobic tail or carboxy terminus end or
portion of cecropin or sarcotoxin. Thus, where lytic
activity is not beneficial or desired, lymphocytes
and/or fibroblast can be stimulated by a shorter
peptide from which the hydrophobic tail has been
removed, i.e. the remaining head including the first
15-25, and preferably the first 18-20 amino acids in
the cecropin or sarcotoxin sequence.

In another embodiment of the invention, a lytic
and/or stimulating peptide is introduced into a higher
animal by placing cells into the animal which have an
expressible gene coding for the peptide. Such cells
may be prepared by genetically transforming cells such
as bone marrow cells, embryos, hematopoietic stem

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cells, and the like. Techniques for such
transformation are well known in the art and include,
for example, transection with retroviral vectors,
electroporation, microinjection and the like.

5   The synthetic peptides of this invention comprise
peptides of greater than or equal to 19 amino acids
having a significant portion of the molecule
amphipathic with a positive charge density over the
amphipathic region.

10   The amino acid homology of peptides can be readily
determined by contrasting the amino acid sequences
thereof as is known in the art. Similarly, the
amphiphilic homology of peptides can be determined by
contrasting the hydophilicity and hydrophobicity of the

15   amino acid sequences. The amino acid sequences of
various preferred lytic peptides are compared in Figure
3.

           Inasmuch as barious changes could be made in the
above methods and composition without departing from

20   the scope of the invention, it is intended that all
matter contained in the above description should be
interpreted as illustrative and not in a limiting
sense.

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Example 1
Synthesis of Lytic Peptides

The lytic peptides were synthesized on a Biosearch Sam Two peptide synthesizer using 4-methyl benzhydrol amine (MBHA) resin with a carboxy-terminal amide. All reagents used in the biosynthesis were obtained from Biosearch, San Rafael, CA.

After extraction and Sephadex column chromatography, the purity of the peptides was determined by high pressure liquid chromatography (HPLC) on Varian 5000 HPLC unit. A Waters Bondepak C18 column, 8 mm x 10 cm Radial-Pak cartridge, employing the Radial Compression Module-100 was utilized. HPLC profiles of the synthesized peptides indicated a purity of more than 95%.

To determine that the syntheses had progressed to completion, amino-terminus sequence analysis was performed on all peptides using an Applied Biosystems 470-A gas phase protein sequencer. PTH-derivitized amino acids generated from the sequencer were analyzed in a Waters Pico Tag system employing a C18 column, 3.8 mm x 15 cm (Waters Nova-Pak).

The lytic peptides of this invention were synthesized by these methods. A listing of the amino acid sequences of these peptides is found in Figure 3.

Example 2
Antibacterial Function of the Lytic Peptides
Shiva-1, LSB-37, and Anubis-8

The antimicrobial activity of the lytic peptides was tested using the MBC and MIC susceptibility tests described by Schoenknecht et al, Manual of Clinical Microbiology, 4th ed., E.H. Lennett et al, eds. Am.

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Soc. for Microbiology, Washington, D.C., p. 1000 (1985), which is hereby incorporated by reference.

Bacteria were subcultured onto Muller-Hinton (MH) agar plates and incubated overnight at 35°C. A tube containing 3 ml Muller-Hinton Broth was inoculated with 5 or more colonies from the plate to achieve a turbidity equivalent of a No. 1 McFarland standard (approximately 10^2 organisms per ml). A volume of 0.1 ml of the turbid inoculum was transferred to 10 ml of MHB and incubated in a shaking water bath at 35°C until turbid. Twofold serial dilutions of the lytic peptides were made in 2 ml of MHB, and the bacteria were standardized to equal 0.5 McFarland turbidity standard (5×10^7 organisms per ml) in a 3 ml of broth. The inoculum was then diluted 1/10 to 5×10^6 organisms per ml. A 100 μl sample of the diluted inoculum was placed into tubes containing serial dilutions of the lytic peptides. The final inoculum of approximately 2.5×10^5 organisms per ml, was then incubated at 35°C for 20 hours. Tubes without visible growth were vortexed vigorously for 15 seconds and reincubated an additional 4 hours. Tubes were then mixed again, and a 0.1 ml sample was spread on the surface of dried Trypticase soy agar plates and incubated overnight at 35°C for MBC determination. After incubation, the number of colonies per plate were counted, and compared with the number of colonies per plate for the original inoculum. The number of colonies that represent 0.1% of the original inoculum was determined, and any number less than or equal to 0.1% of the original inoculum was considered to be a 99.9% bactericidal kill.

An additional dilution of the inoculum in MHB to achieve a concentration of 5×10^2 organisms per ml was made, and 0.1 ml of this suspension was spread onto MH agar plates with a sterile, bent glass rod. The

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samples were prepared in duplicate and incubated overnight at 35°C. The next day, the lowest concentration of the lytic peptide at which no growth of bacteria was detected on the agar plates was
5   recorded as the MIC.

To a 1.5 ml Eppendorf tube was added 50 μl of the bacteria under investigation and 50 μl of 0.01 M phosphate buffered saline (PBS), pH 7.0. The bacterial cells were collected in late log phase growth and
10   incubated at room temperature for one hour and then at 37° for 30 minutes in the presence of 0.01-10μM lytic peptide. At the end of the incubation, the treated bacteria were diluted 1:1000 and 100 μl of this dilution was plated onto Tryptose agar growth plates.
15   The plates were then incubated for three days at 37° C. The test plates were then examined, colony counts were made, and MBC values calculated.

An MBC value less than 50μM is indicative of an effective antimicrobial agent.
20   The results of treatment with the lytic peptides LSB 37, Shiva-1, Anubis-8 and Vishnu-3 on the gram negative (-) bacteria E. Coli, P. aeruginosa , and the gram positive (+) bacteria S. aureus are shown in Table I.

25

TABLE I

	E. coli (-)	P. aeruginosa (-)	S. aureus (+)
LSB-37	16 μM	8 μM	64 μM
Shiva-1	2 μM	4 μM	32 μM
Anubis-8	16 μM	16 μM	8 μM
Vishnu-3	> 64	> 64	> 64

30

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Example 3

Antibacterial Functions of the

Lytic Peptides LSB-37 and Anubis-2

The procedure of example 2 was followed using a variety of freshly isolated bacterial samples. The MIC and MBC results are indicated in Table II.

TABLE II

| | LSB-37 | | Anubis-2 | |
	MIC (µM)	MBC (µM)	MIC (µM)	MBC (µM)
Bacteria				
Acinetobacter Cal Anit	0.5	1	3.2	3.2
Citrobacter Diversus	1	4	3.2	6.4
Citrobacter Freundii	1	4	3.2	6.4
Enterobacteria Cloacae	2	8	6.4	12.8
Eschera Coli	1	4	6.4	12.8
Klebsiella pheumoniae	2	8	3.2	6.4
Klebsiella pheumoniae-2	4	8	6.4	25.6
Morganella morganil	32	-	51.2	-
Pseudomonas aeruginosa1	8	16	6.4	25.6
Pseudomonas aeruginosa2	4	16	3.2	6.4
Pseudomonas aeruginosa3	8	16	6.4	51.2
Serratia Marcescens	32	-	51.2	-
Gram Positive Bacteria				
Enterococcus-1	8	32	1.6	3.2
Enterococcus-2	32	-	51.2	-
Enterococcus-3	32	-	51.2	-
Staph aureus	32	-	6.4	51.2
Staph Coag neg	32	-	1.6	3.2
Staph aureus-MR1	n.a.	-	51.2	-
Staph aureus-MR2	n.a.	-	12.8	25.6

- = Data not available.

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Example 4
Lytic Peptides Lyse Bacteria

The method of example 2 was followed to test the effectiveness of the synthetic lytic peptides on bacterial cells. The results are shown in Table III, and represent MBC values (μM). A MBC value > approximately 50 μM is considered an effective bacterial inhibitor.

TABLE III

	E. coli (-)	P. aeuriginosa (-)	S. aureus(+)
Cecropin-B	4.5	4.2	68
SB-37	2.0	3.5	64
LSB-37	2.0	3.6	64
Shiva-1	4.0	4.5	64
Shiva-10	1.0	2.0	NA
Manitou-1	8	16	32
Hecate-1	4.0	10.0	NA

Example 5
Treatment of Bacterial Infection in Catfish
By Injection of Synthetic Lytic Peptides

Catfish fingerlings weighing approximately 2 oz. were pretreated with 5×10^6 cells of the bacteria Edwardsiella ictaluri. Treatment groups of the fish were then injected intraperitoneally (i.p.) with the lytic peptide LSB-37 dissolved in saline, or a saline control once per day for a period of 4 days. The survival rates and the bacteria counts in the liver of the autopsied fish indicated that the lytic peptide LSB-37 was successful in reducing the lethal effects of E. Ictaluri bacterial infection in catfish. These data are shown in Table IV.

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TABLE IV

LSB-37 Treatment of Catfish Fingerlings
Infected With Edwardesiella Ictaluri

Treatment	% Survival	# Bacteria/g Liver
Non-infected Saline control	85	N.A.
Non-infected + 200 μg LSB-37	100	N.A.
Infected Saline controls	10	4.8×10^6
Infected 20 μg LSB37	25	4.2×10^6
Infected 100 μg LSB-37	30	2.1×10^6
Infected 200 μg LSB-37	55	0.8×10^6

Example 6

Synthetic Lytic Peptides Lyse Cancer Cells

EL-4 lymphoma cells obtained from ATCC were suspended in RPMI 1640 growth medium at a density of 5×10^6 cells per 100 μl in microtiter test plates. Various concentrations of lytic peptides in a total volume of 10 μl were added, and the test plates incubated at 37°C in 5% CO for one hour. Cell viability was then determined by trypan blue exclusion. The results shown in Table V indicate synthetic lytic peptides kill cancer cells.

TABLE V

% EL-4 Cells Killed

Concentration (μM)	LSB-37	LSB*37	Shiva-1	Manitou-1
10	N.D.	N.D.	2	5
25	2	2	18	28
50	5	10	42	78
100	38	45	87	100

B-16 myeloma cells and normal 3T3 human fibroblast were either permitted to attach to the test plate, or treated with trypsin to free the cells from the plastic

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dish prior to treatment. Cells were then treated with 100 M lytic peptide for one hour as described above, and cell viability was then determined by trypan blue exclusion. The results are shown in Table VI.

5

TABLE VI

% B-16 Cell Viability

	Control	LSB-37	Manitou-1
3T3 Monolayer	96	94	85
B-16 Monolayer	94	78	19
3T3 Suspension	98	12	5
B-16 Suspension	96	22	0

These data indicate both Manitou-1 and LSB-37 are able to lyse trypsinized normal 3T3 Fibroblast and B-16 cancer cells, in suspension culture. However, when cells are attached to the growth plate in a monolayer culture, Manitou-1 is more effective than LSB-37 in selectively killing cancer cells. The killing of normal 3T3 fibroblast in suspension culture is thought to be due to disruption of cell membrane by treatment with trypsin.

20

Example 7
The Role of Cytoskeletal
Structure in Susceptibility To Lysis

Primary fetal donkey dermal cells (FDD) were treated to assess the effects of cold and cytoskeletal inhibitors on cellular resistance to lytic peptides, to substantiate the hypothesis that a weakened cytoskeletal system renders

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a normally resistent cell susceptible to the lytic activities of the lytic peptides.

In the first treatment group, FDD were chilled to 4°C for 15 minutes and then exposed to 100 μM lytic peptide for an additional 15 minutes at 4°C. The cells were then washed, and incubated for 45-60 minutes in fresh DMEM medium.

A second test group of FDD was placed in medium containing either cytochalasin-D (5 μg/ml) or colchicine (0.1 μg/ml) and incubated at 37°C for 15 minutes. The cells were then exposed to 100 μM lytic peptide for an additional 15 minutes, washed and incubated 45-60 minutes in fresh DMEM medium at 37°C.

The third test group of FDD cells was incubated for 15 minutes at 37°C, exposed to 100 M lytic peptide for 15 minutes, washed, and incubated an additional 45-60 minutes in fresh DMEM medium at 37°C. At the end of the incubation period, cell counts were made by trypan blue exclusion, and the percent viability calculated. The results are shown in Table VIII, and indicate that treatment with the cytoskeletal inhibitors colchicine and cytochalasin D, or cold treatment significantly enhances the lytic ability of the peptides.

Table VII
% Viability of Treated DD Cells

Treatment Group	LSB-37	Manitou-1
Control, no peptide	98	96
Warm-treated	96	78
Coldtreated	70	8
Colchicine-treated	40	30
Cytochalasin D-treated	32	23

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Example 8
Proliferative Activity of Synthetic Lytic Peptides

Human mononuclear leukocytes were isolated from whole blood in the following manner. Blood was collected in vacutainer tubes containing EDTA, and was then diluted with an equal volume of calcium and magnesium-free PBS and layered over Histopaque resin (GIBCO). The tubes were centrifuged for 30 minutes at 25° C and 450 x g. The plasma and platelets above the banded layer of cells in the resulting gradient were removed and discarded. The banded white blood cells were removed to fresh test tubes, washed 3 times with PBS, and resuspended in RPMI medium containing 10% fetal bovine serum (FBS), mercaptoethanol, and penicillin and streptomycin as antibiotics. The cells were then counted and resuspended to a final concentration of 3×10^6 cells/ml.

To each well of a 96 well flat-bottomed microtiter plate was added 100 μl of cells (3×10^5 cells). Treatment groups were set in quadruplicate samples. The appropriate concentration of test peptide and/or the mitogen hytohemaglutinate (PHA), was added in a total volume of 100 μl of medium to the test wells. The plates were then incubated at 37° C and 5% CO2 for a duration of 72 hours. At the end of the incubation period, 1 μCi of 3H-thymidine in 1 μl TRIS-HCI, PH8 was added to each well, and the plates incubated an additional 18 hours.

Cellular DNA was harvested onto glass fiber filters using a semi-automatic cell harvester. The glass filters were then placed into vials containing scintillation fluid, and the samples were counted in a scintillation counter. The uptake of 3H-thymidine is an indicator of cell proliferation, thus the higher the number of counts in the sample, the greater the proliferative activity of the cells. The results in Table XI indicate that low dose of the synthetic peptides of this invention stimulate cell

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growth. The data also suggest that the lytic peptides may interact synergistically with other chemical agents, such as PHA to enhance their activity.

Table VIII
^{3}H-Thymidine Uptake in WBC
% of Control

| | | PHA (mg) | | |
		0.0	0.5	5.0
Manitou-1	10	239	191	125
(μM)	25	325	153	143
	50	62	61	63
	100	39	8	0
Anubis-2	10	149	290	120
(μM)	25	46	393	108
	50	19	4	1
	100	26	5	0

Example 9
Proliferation of Lymphocytes by Treatment with Lytic Peptides and Synergistic Activity with Tetanus Toxin

White blood cells were isolated as described in Example 8 from a person who had received a tetanus shot during the previous 6 months. It was expected that some of the lymphocytes would thus respond to the exogenous tetanus toxin with proliferation. The lymphocytes were prepared and incubated as described for example 8 in the presence of the appropriate concentration of Tetanus Toxin (0 - 4.0 μg/μl) and/or lytic peptide (10 - 100 μM). At the end of the incubation period, cells were counted by trypan blue exclusion, and the data expressed as percent of

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control (no treatment). These data are shown in Table IX, and indicate that the peptide Vishnu-3, which is devoid of lytic activity, is a potent stimulator of cell proliferation. These data also suggest that the lytic peptides may be utilized as general adjuvants.

TABLE IX
PROLIFERATION OF LYMPHOCYTES (% OF CONTROL)

		Tetanus Toxin (μg/l)				
		0	0.5	1.0	2.0	4.0
Vishnu-3 (μM)	10	170	50	125	155	110
	25	210	155	145	150	140
	50	160	150	140	135	135
	100	200	175	140	165	155
LSB-37 (μM)	10	150	170	145	130	140
	25	170	150	190	140	170
	50	120	220	240	250	225
	100	50	50	50	50	50

Example 10
Synergy of Lytic Peptides With Antibiotics

The synergy of the lytic peptides was examined by incubating bacterial cells in the presence of both a lytic peptide and a known antibiotic according to the methods described for Example 2. At the end of the incubation period, cell counts were made, and the percent viability of the bacterial cells was calculated. The results are shown in Table X, and suggest synergistic action of Manitou-1 and streptomycin in E. Coli.

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TABLE X

% Cell Death In E. coli

Antibiotic (µM)	Manitou-1 (µM)			
	0.0	0.01	0.10	1.0
Control -0	0	0	0	0
Tetracycline - 0.5	0	68	0	0
Tetracycline - 5.0	58	88	58	47
Streptomycin - 0.5	0	99.9	68	0
Streptomycin - 5.0	87	58	99.9	99.9

% Cell Death In S. auerus

Antibiotic (µM)	Manitou-1 (µM)			
	0.0	0.01	0.10	1.0
Control - 0	0	0	0	0
Tetracycline - 0.5	0	0	0	0
Tetracycline - 5.0	35	35	35	35
Streptomycin - 0.5	0	0	0	0
Streptomycin - 5.0	87	87	95	86

Example 11

Wound Healing

To evaluate the ability of the lytic peptide LSB-37 to limit the growth, as well as the septicemic disease associated with Pseudomonas aeurginosa infection, the following protocol was followed.

Thirty-five Sprague Dawley female rats weighing between 200 and 300 grams were anesthetized, the hair on their backs was clipped, and they were secured in an apparatus which allowed for selective exposure of approximately 20% of their skin surface area to a 10 second exposure to boiling water. This model, developed by Walker

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and Mason (Journal of Trauma, Vol. 8, pages 1049-1051, 1968) is an established standard procedure in burn trauma research. The model consistently results in uniform full thickness skin burn.

Immediately following the burning, rats were divided into seven test groups of five rats each, and housed in seven cages. In six groups, the burned skin was seeded with 1.0 ml of PBS containing 1×10^6 cells of Pseudomonas aeruginosa (supplied by Dr. A.T. McManus, Chief, Microbiology Branch, U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas). The seventh group served as non-infected and non-treated control.

In group one, the burned and seeded skin was treated with 0.5 ml of 100 μM of LSB-37 in PBS. The LSB-PBS was sprayed onto the burned area, and gently rubbed into the clipped hair by a gloved finger. These rats were also inoculated intramuscularly in the thigh muscles of both right and left legs (alternating) with a dose of LSB-37 in PBS equal to 5.0 mg of peptide per pound of body weight. The intramuscular injections were begun 20 hours post burn infection, and were repeated every six hours for three days.

Group two rats received 0.5 to 1.0 ml of PBS sprayed on and rubbed into the burned areas. The burned and seeded skin of group three rats was treated with 1% silver sulfadizaine cream (Tine SSD S-901842- Exp. March,89). The cream was digitally applied to the burn at a thickness of approximately 1.0 mm. The burned and seeded skin of group four rats was treated topically only with LSB-37 beginning at nine hours post burn-infection. All treatments were begun at nine hours post burn-infection, and repeated every six hours for three days.

Of the 35 test rats, five did not recover from the general anesthesia. Three additional rats in the control group (burned, but not infected nor treated) died. These

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rats were replaced with three additional rats which were then maintained in a separate cage for the duration of the experiment. One animal in group four and one in group six did not recover from the anesthesia. Several rats were moribund, or had been traumatized by cannibalism and were euthanatized by an overdose of ether anesthetic. The following table illustrates the number of surviving animals.

TABLE XI

ANIMALS SURVIVING BURN TREATMENT

Burned, not infected	5
Topical PBS	0
Topical Silver Sulfadiazine	5
Topical LSB-37 at 9 hours	1
Topical LSB-37 at 20 hours	1
I.M. LSB-37 at 20 hours	1
Topical at 9; I.M. at 20 hours	0

The high mortality rate due to cannibalism, failure to recover from the anesthesia, and secondary infection by gram positive bacteria make it difficult to draw conclusions from the numbers generated. However, the dramatic recovery of the wound in the live animals treated with the lytic peptide LSB-37 suggests a profound effect of this peptide on would healing. Photographs shown in Figure 8 of surviving animals clearly show the animals treated either topically or by injection with LSB-37 have vastly accelerated regeneration of the tissue over the wound, with less scarring, and accelerated hair growth.

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CLAIMS:

1   1.   A peptide comprising:

2   (a)   an α-helical conformation of approximately
3   15 to 40 amino acids;

4   (b)   one or more sets of 18 sequential amino
5   acids, each amino acid spaced 20 apart about the axis of
6   the helix, exhibiting approximately 60-100% aligned
7   amphipathy;

8   (c)   four or more positively charged amino acids;

9   (d)   where the amino acid at position 19 is not
10   glutamate or aspartate, and the amino acid at position 23
11   or 24 is not proline; and

12   (e)   where no four consecutive amino acids are
13   of the sequence: lysine - arginine - lysine - arginine.

1   2.   The peptide of claim 1, wherein the peptide is
2   comprised of less than 20 amino acids.

1   3.   The peptide of claim 1, wherein only one set of
2   18 sequential amino acids exhibit aligned amphipathy.

1   4.   The peptide of claim 3, wherein the set of 18
2   sequential amino acids exhibiting amphipathy is located at
3   the carboxy terminal half of the peptide.

1   5.   The peptide of claim 3, wherein the set of 18
2   sequential amino acids exhibiting amphipathy is located at
3   the amino terminal half of the peptide.

1   6.   The peptide of claim 1, wherein said peptide is
2   comprised of combinations of 3 to 5 amino acids.

1   7. The peptide of claim 6, wherein said amino acids
2 are selected from the group comprising: alinine, leucine,
3 phenylalanine, lysine, and glutamine.

1   8.   The polypeptide of claim 2, wherein said
2 polypeptide has the amino acid sequence:
3   LPKWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG

1   9.   The polypeptide of claim 2, wherein:
2   said polypeptide has the amino acid sequence:
3 MKRMVSWSFKKLKTMKKLLLLLLCVFLVKS

1   10. The polypeptide of claim 2, wherein:
2   said polypeptide has the amino acid sequence:
3 MKRMVSWSFRKLKTMKRLLLLLLCVFLVKS

1   11. The polypeptide of claim 2, wherein:
2   said polypeptide is comprised of the amino acid
3 sequence:
4   FAKKLAKKLKKLAKKLAKLALAL

1   12. The polypeptide of claim 2, wherein:
2   said polypeptide is comprised of the amino acid
3 sequence:
4   FAKKLAKKLKKLAKKLAKLALALKALALKAL

1   13. The polypeptide of claim 2, wherein:
2   said polypeptide is comprised of the amino acid
3 sequence:
4   FAKKLAKKLKKLAKKLAKLALALKALALKALALKALAL

1   14. The polypeptide of claim 3, wherein:
2   said polypeptide is comprised of the amino acid
3 sequence:
4   FALALKALKKALKKLKKALKKAL

1   15. The polypeptide of claim 3, wherein:
2   said polypeptide is comprised of the amino acid
3   sequence:
4   FAKLALAKLALALKALKKALKKLKKALKKAL

1   16. The polypeptide of claim 3, wherein:
2   said polypeptide is comprised of the amino acid
3   sequence:
4   FALAKLALAKLALAKLALALKALKKALKKLKKALKKAL

1   17. The polypeptide of claim 1, wherein:
2   said polypeptide is comprised of the amino acid
3   sequence:
4   FAKKLAKLAKKLAKLAKKLAKKL

1   18. The polypeptide of claim 1, wherein:
2   said polypeptide is comprised of the amino acid
3   sequence:
4   FAKKLAKKLKKLAKKLAKLAKKL

1   19. The polypeptide of claim 1, wherein:
2   said polypeptide is comprised of the amino acid
3   sequence:
4   FAKKLAKKLKKLAKKLAKLAKKLAKKLKKLA

1   20. The polypeptide of claim 1, wherein:
2   said polypeptide is comprised of the amino acid
3   sequence:
4   FAKKLAKKLKKLAKKLAKLAKKLAKKLKKLAKKLAKLA

1   21. The polypeptide of claim 1, wherein:
2   said polypeptide is comprised of the amino acid
3   sequence:
4   QFAQKLAKLAQQLAKKLQQLAKK

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1   22. The polypeptide of claim 1, wherein:

2   said polypeptide is comprised of the amino acid

3 sequence:

4   QLQAKLKAQLQAKLKAQLQAKLK

1   23. The polypeptide of claim 1, wherein:

2   said polypeptide is comprised of the amino acid

3 sequence:

4   EFAEKLAKLAEELAKKLEELAKK

1   24. The polypeptide of claim 1, wherein:

2   said polypeptide is comprised of the amino acid

3 sequence:

4   LKKLAKLAKKLAKLAKKLAKK

1   25. The polypeptide of claim 4, wherein:

2   said polypeptide is comprised of the amino acid

3 sequence:

4   MPKWKVFKKIEKVGRNIRN

1   26. The polypeptide of claim 4, wherein:

2   said polypeptide is comprised of the amino acid

3 sequence:

4   MPRWRLFRRIDRVGKQIKQ

1   27. The polypeptide of claim 4, wherein:

2   said polypeptide is comprised of the amino acid

3 sequence:

4   MPKEKVFLKIEKMGRNIRN

1   28. The polypeptide of claim 4, wherein:

2   said polypeptide is comprised of the amino acid

3 sequence:

4   MKRMVSWSFKKLKTMKKLL

- 43 -

1   29.   A method of killing microbial cells comprising:

2   contacting said pathogenic microorganisms with
3   an effective dose of a therapeutic composition comprising
4   the polypeptide of claim 1.

1   30.   The method of claim 29 wherein:
2   said contacting is with a therapeutic composition
3   comprising human beta fibrin signal peptide.

1   31.   The method of claim 30 wherein:
2   said pathogenic microorganisms are selected from
3   the group comprising: bacteria, fungi, yeast and protozoa.

1   32.   The method of claim 31 wherein:
2   said contacting is with a therapeutic composition
3   comprising human beta fibrin signal peptide.

1   33.   The method of claim 32 wherein:
2   said bacteria are selected from gram positive
3   and gram negative bacteria.

1   34.   The method of claim 33 wherein:
2   said contacting is with a therapeutic composition
3   comprising human beta fibrin signal peptide.

1   35.   A method of treating microbial infection in
2   plants and animals comprising:
3   administering to the plants or animals an
4   effective dose of a therapeutic composition comprising the
5   polypeptide of claim 1.

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1   36. The method of claim 35, wherein:
2   said administering is with a therapeutic
3   composition comprising human beta fibrin signal peptide.

1   37. The method of claim 35 wherein:
2   said microbial infection is selected from the
3   group comprising: bacterial, viral, fungal, yeast or
4   protozoan infection.

1   38. The method of claim 35 wherein:
2   said administering is by means selected from the
3   group comprising: topical application, injection and
4   infusion.

1   39. A method for the treatment of cancer in animals
2   comprising:
3   administering to said animals an effective dose
4   of a therapeutically active composition comprising the
5   polypeptide of claim 1.

1   40. The method of claim 39 wherein:
2   said administering is with a theraupeutic
3   composition comprising human beta fibrin signal peptide.

1   41. A method for the prevention of a pathogenic
2   disease in plants and animals comprising:
3   incorporating into the plant or animal's genome
4   one or more genes encoding the polypeptide of claim 1.

1   42. The method of claim 41, wherein:
2   said administering is with a therapeutic
3   composition comprising human beta fibrin signal peptide.

- 45 -

1   43. · A method for the proliferation of cell growth
2   comprising:
3   contacting the cells with an effective dose of
4   a therapeutic composition comprising the polypeptide of
5   claim 1.

1   44. The method of claim 43 wherein:
2   said contacting is with a therapeutic composition
3   comprising human beta fibrin signal peptide.

1   45. The method of claim 43, wherein:
2   said cells are selected from the group comprising
3   white blood cells and fibroblast.

1   46. A method for the healing of wounds comprising:

2   administering to the wound an effective dose of
3   a therapeutic composition comprising the polypeptide of
4   claim 1.

1   47. The method of claim 46, wherein:
2   said administering is with a therapeutic
3   composition comprising human beta fibrin signal peptide.

1   48. A method to facilitate the action of therapeutic
2   agents comprising:
3   administering said therapeutic agents in a
4   composition comprising an effective dose of the polypeptide
5   of claim 1.

1   49. The method of claim 48, wherein:
2   said administering is with a therapeutic
3   composition comprising human beta fibrin signal peptide.
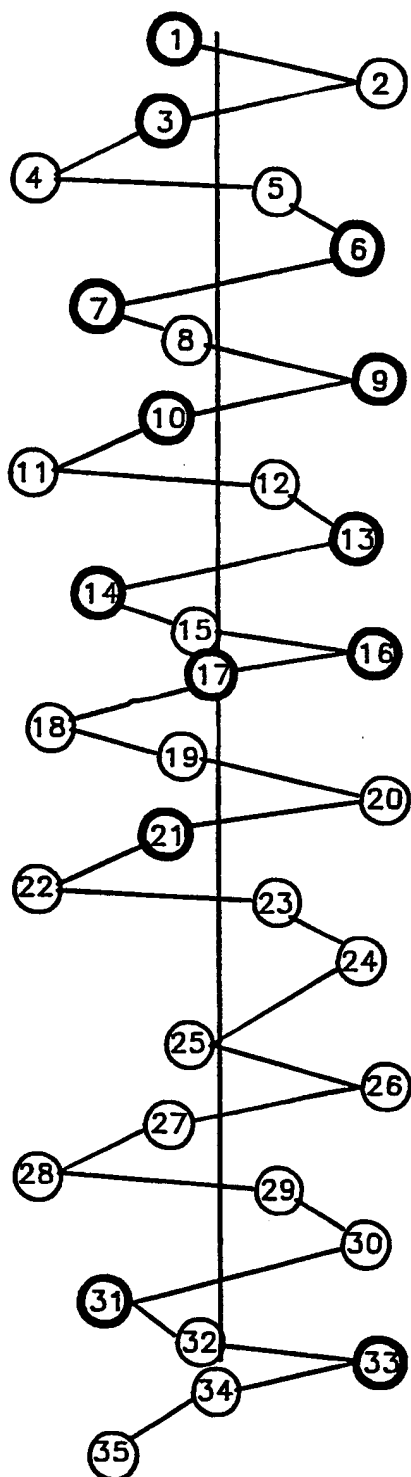
NATURAL CECROPIN B HELICAL WHEEL

○ HYDROPHOBIC   ◉ HYDROPHILIC

FIG. 1a

FIG. 1b

FIG. 1c

HIGH DENSITY OF + CHARGE

AMINO TERMINUS

CARBOXY TERMINUS
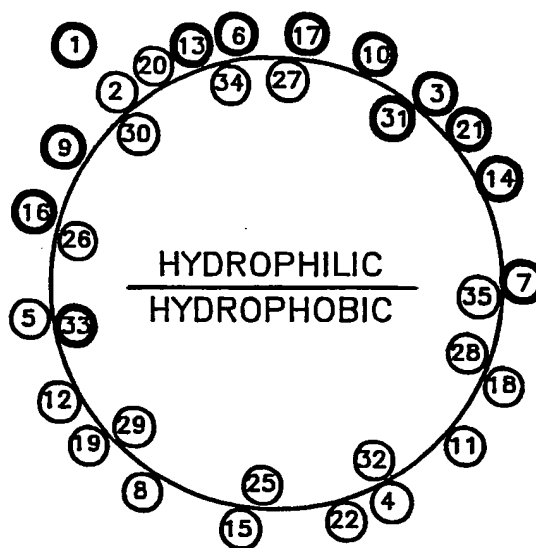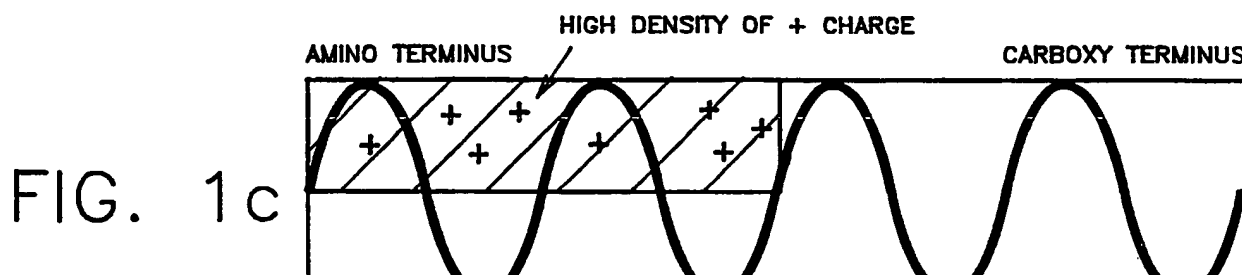
CLASSES OF LYTIC PEPTIDE ANALOGS

FIG. 2a

SHIVA SERIES AND MANITOU SERIES

FIG. 2b

HECATE SERIES

FIG. 2c

ANUBIS SERIES

FIG. 2d

VISHNU SERIES

Name	Sequence	# A.A.
Cecropin B	.KWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKAL	35
SB-37	MP.KWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG	38
LSB-37	LP.KWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG	38
SB*37	MP.KEKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG	38
SHIVA-1	MP.RWRLFRRIDRVGKQIKQGILRAGPAIALVGDARAVG	38
SHIVA-10	.FAKKLAKKLKKLKKLAKKLAKLAL	23
SHIVA-11	.FAKKLAKKLKKLKKLAKKLAKLALAL	31
SHIVA-12	.FAKKLKKLKKLAKKLALALALKALALALKALAL	38
β-FIBRIN SIGNAL	.MKRMVSWSFHKLKTMKHLLLLLLLCVFLVKS	30
MANITOU-1	.MKRMVSWSFKKLKTMKKLLLLLCVFLVKS	30
MANITOU-2	.MKRMVSWSFRKLKTMKRLLLLLLCVFLVKS	30
HECATE-1	.FALALKALKKALKKALKKAL	23
HECATE-2	.FAKLALKKALALKKALKKKAL	31
HECATE-3	.FALAKLAIAKLAKKLKKALKKKLALKKAL	38
ANUBIS-1	.FAKKLAKLAKKLAKKLAKKL	23
ANUBIS-2	.FAKKLKKLKKLAKKLAKKL	23
ANUBIS-3	.FAKKLAKLAKKLAKKLAKKLA	31
ANUBIS-4	.FAKKLKKLAKKLAKKLKKLAKKLA	38
ANUBIS-5	.QAFQKLAKLQQLAKKLQQLAKK	23
ANUBIS-8	.LKKLAKKLAKKLAKKLAKK	21
VISHNU-1	MP.KWKVFKKIEKVGRNIRN	19
VISHNU-2	MP.RWRLFRRIDRVGKQIKQ	19
VISHNU-3	MP.KEKVFLKIEKMGRNIRN	19
VISHNU-8	.MKRMVSWSFKKLKTMKKLL	19

LYTIC PEPTIDE AMINO ACID SEQUENCES

FIG. 3

CECROPIN B

SB−37

LSB−37

SB+37

SHIVA−1

SHIVA−10

SHIVA−11

SHIVA−12

β−FIBRIN S.P.

MANITOU−1

MANITOU−2

HECATE−1

HECATE−2

HECATE−3

ANUBIS−1

ANUBIS−2

ANUBIS−3

ANUBIS−4

ANUBIS−5

ANUBIS−8

VISHNU−1

VISHNU−2

VISHNU−3

VISHNU−8
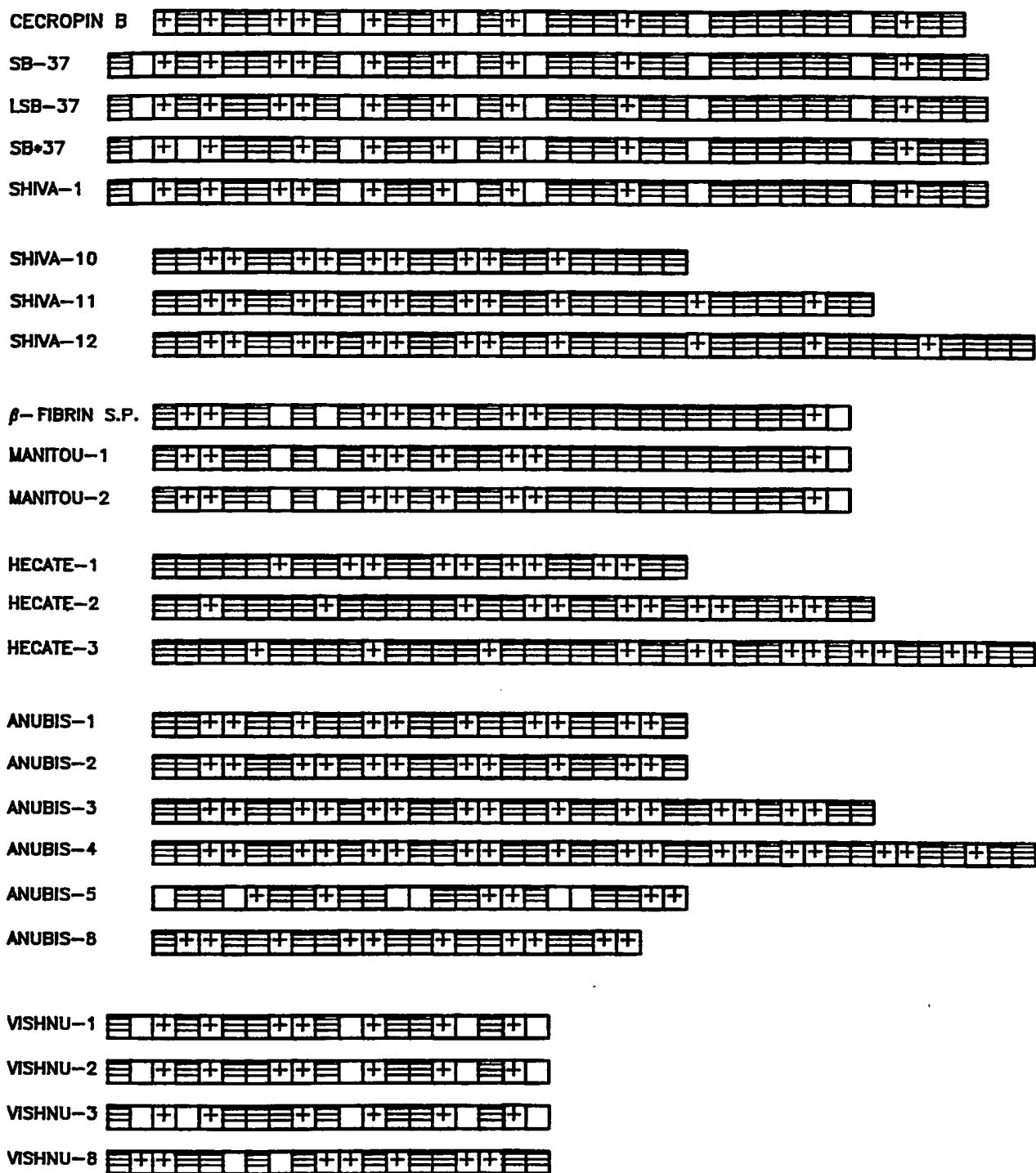
Hydrophobicity/Amphipathy/Charge Distribution

FIG. 4
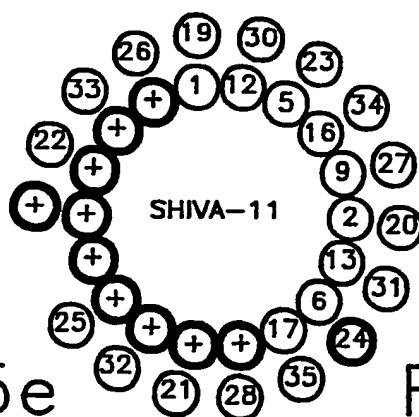
○ Hydrophobic   ● Hydrophilic

FIG. 5a — LSB-37

FIG. 5b — SB*37

FIG. 5c — SHIVA-1

FIG. 5d — SHIVA-10

FIG. 5e — SHIVA-11

FIG. 5f — SHIVA-12

FIG. 5g — β-FIBRIN SIGNAL PEPIDE

FIG. 5h — MANITOU-1
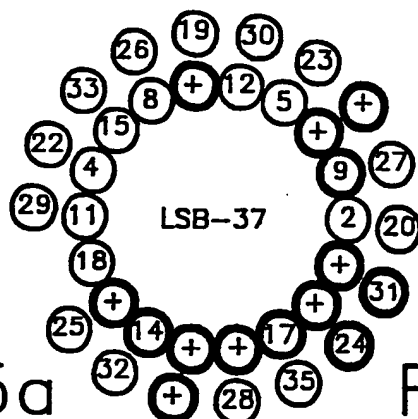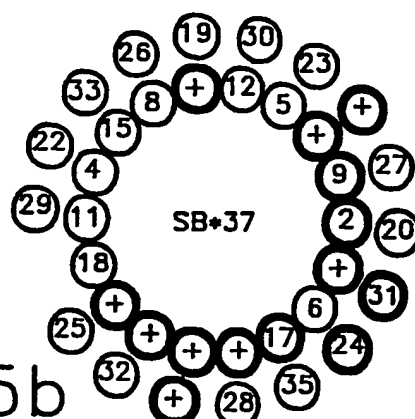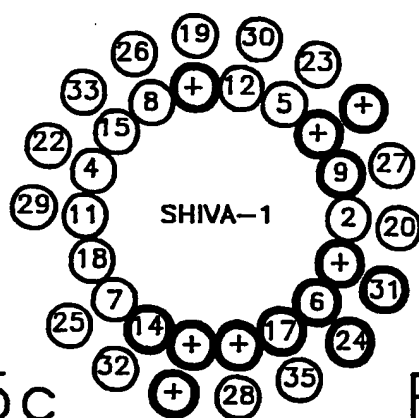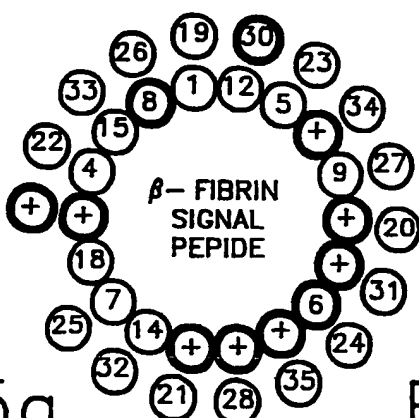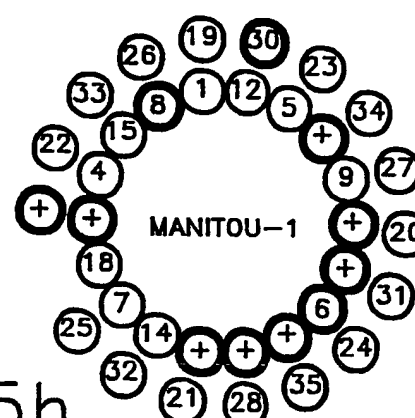
○ Hydrophobic   ○ Hydrophilic
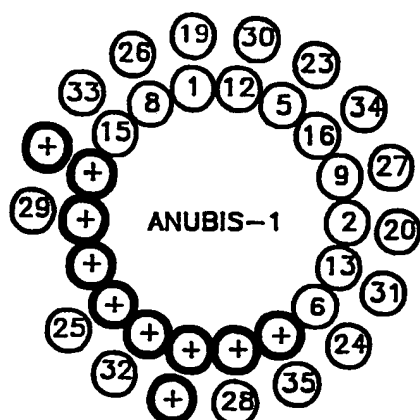
FIG. 6a
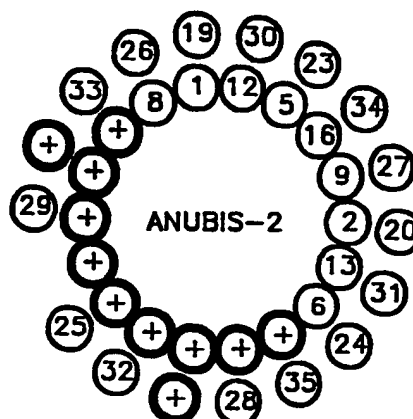
FIG. 6b
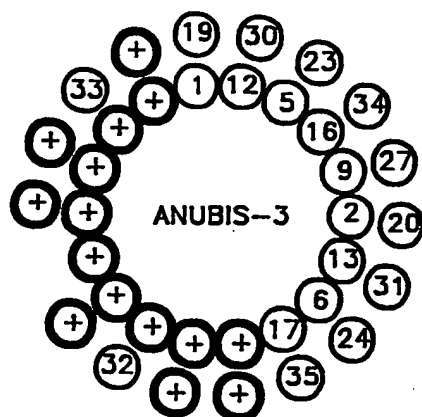
FIG. 6c
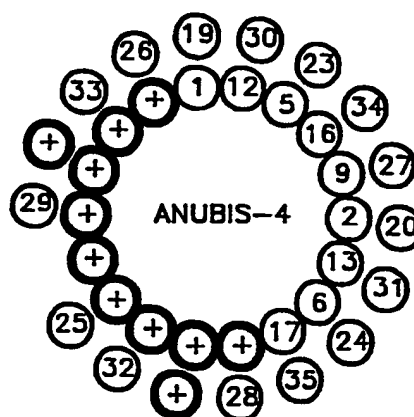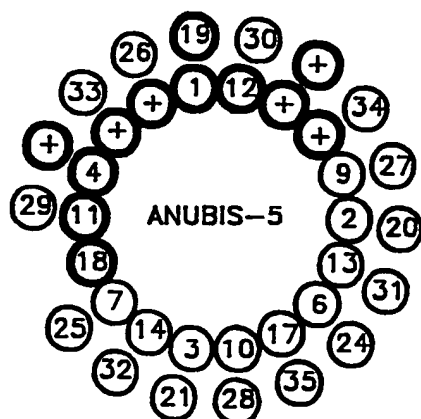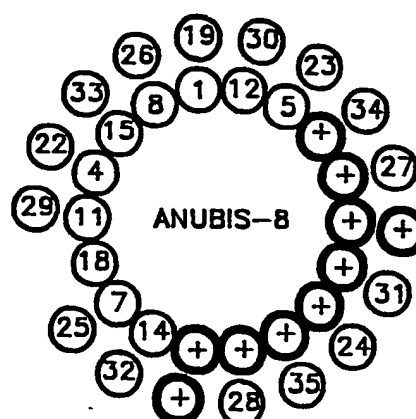

FIG. 6d

FIG. 6e

FIG. 6f

○ Hydrophobic   ● Hydrophilic

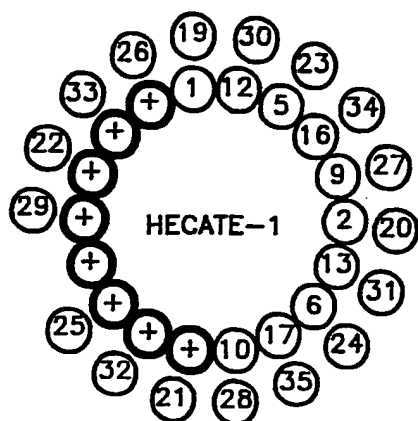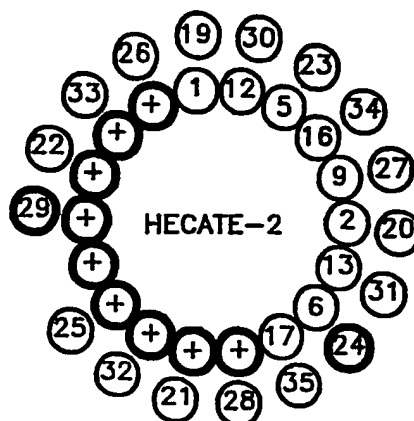NOTE: 1 IS CARBOXY TERMINUS

FIG. 7a

FIG. 7b

FIG. 7c

FIG. 7d

LEFT — BURNED; INFECTED; TREATED WITH CYTOLYSIN
BY INJECTION
RIGHT — BURNED; NOT INFECTED (CONTROL)

FIG. 8a

LEFT — BURNED; INFECTED; STANDARD SILVER SULFADIAZINE
TREATMENT
RIGHT — BURNED; INFECTED; TREATED TOPICALLY WITH
CYTOLYSIN

FIG. 8b

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 1/06; A61K 37/02; A61K 48/00

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
US	435/259 530/324 530/326

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 5

CAS, MEDLINE, Biosis, APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X	Biochimica et Biophysia Acta, 864, issued 1986, BERNHEIMER, ET AL., "Interactions between membranes and cytolylic peptides", pages 123–141. See pages 127,128,131.	1,3,4,14–16
Y	Proc. Natl. Acad. Sci., Vol. 85, issued July 1988, CHRISTENSEN, ET AL., "Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes", pages 5072–5076. See abstract introduction, fig. 1.	1,17–24
X	Biochemistry, Vol. 24, No. 7, issued 1985, ANDREU, ET AL., "N-Terminal Analogues of Cecropin A: Synthesis, Antibacterial Activity, and Conformation Properties", pages 1683–1688. See entire article.	1,3,5,8–13 and 25–28
Y	US, A, 4,355,104, (HULTMARK, ET AL.) 19 October 1982. See Columns 6–10.	28–40,46–49
Y	Biochemical and Biophysical Research Communications, Vol. 87, No. 1, issued 15 March 1979, BESSLER, ET AL., "Interaction of Membrane Modifying Peptide Antibiotics From Trichoderma Viride With Leukocytes", pages 99–105. See abstract, page 99, introduction, discussion.	28–40,43–45

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
30 JULY 1990	17 SEP 1990

International Searching Authority 1	Signature of Authorized Officer 20
ISA/US	NGUYEN NGOC-BO INTERNATIONAL DIVISION SUZANNE ZISKA

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	The Journal of Biological Chemistry, Vol. 262, No. 1, 05 February 1987, NAKAJIMA, ET AL., "Interaction between Liposomes and Sarcotoxin IA, a Potent Antibacterial Protein of Sarcophaga peregrina (Flesh Fly)", pages 1665-1669. See page 1668, Discussion.	28-40
Y	Virology Vol. 143, issued 1985, RENNELL, ET AL., "Phage P22 Lysis Genes: Nucleotide Sequences and Functional Relationships with T4 and λ Genes", pages 280-289. See page 286, 2ND Col. and page 287 top.	28-40

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter ¹ not required to be searched by this Authority, namely:

2.☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹, specifically:

3.☐ Claim numbers............, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers ┄ ┄searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international ┄ ┄┄ ┄rs only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search r┄┄ ┄s r┄┄┄ ┄ to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Autho┄ ┄ ┄ ┄id not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

09363884 97255877 PMID: 9101208
  Influence of urological morbidity on quality of life in patients with prostate cancer .
  Fossa S D; Woehre H; Kurth K H; Hetherington J; Bakke H; Rustad D A; Skanvik R
  Norwegian Radium Hospital, Oslo, Norway.
  European urology (SWITZERLAND) 1997, 31 Suppl 3 p3-8, ISSN 0302-2838
Journal Code: 7512719
  Document type: Journal Article
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: Completed
  Subfile: INDEX MEDICUS

  OBJECTIVE: To evaluate the prevalence of urological morbidity in patients with prostate cancer and its influence on global quality of life (QL). METHODS: 379 patients with prostate cancer completed several questionnaires (EORTC QLQ-C33, I-PSS, PAIS [sexuality]) and were grouped according to the type of treatment they had undergone: observation only (n = 57), androgen deprivation (n = 112), radiotherapy (n = 114) and radical prostatectomy (n = 96). A group of 86 urologists also completed the questionnaires, imagining that they had stable prostate cancer and had been undergoing androgen deprivation for at least 1 year. Their responses were compared with those from patients who had undergone androgen deprivation. RESULTS: In all patient groups urinary symptoms were mostly mild to moderate but 12% of patients who had undergone radiotherapy and 11% of hormonally treated patients described severe symptoms. Severe urinary leakage occurred in 16% of patients who had undergone radical prostatectomy. 22% of all patients were impotent before treatment ; after treatment sexual life was considerably disturbed in all patients except those who had undergone observation only. Fatigue was a general problem in all patients. In the logistic regression analysis fatigue and lower urinary tract symptoms (LUTS), but not disturbance of sexual life or urinary leakage, were correlated with global QL. In the doctor-patient comparison, doctors underestimated the disturbance of sexual life in patients who had undergone androgen deprivation therapy but overestimated the impairment of QL and psychological distress experienced by these patients. CONCLUSIONS: LUTS and fatigue are independent predictive factors for global QL after initial treatment in patients with prostate cancer . Radiotherapy of the prostate leads to more significant morbidity than was generally anticipated by doctors. The disagreement between doctors' and patients' assessments of QL following treatment indicates the necessity for further systematic QL evaluation in these patients.